DESCRIPTION

INACTIVATION OF HIV CO-RECEPTORS AS THERAPY FOR HIV INFECTION

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the fields of HIV infection. More particularly, it concerns novel methods and compositions for the treatment of HIV infection and methods for conferring HIV resistance.

2. Description of Related Art

Human Immunodeficiency Virus-1 (HIV-1) infection has been reported throughout the world in both developed and developing countries. HIV-2 infection is found predominately in West Africa, Portugal and Brazil. It is estimated that as of 1990 there were between 800,000 and 1.3 million individuals in the United states that were infected with HIV. An important obstacle to developing a vaccine against HIV is that the mechanism of immunity to HIV infection is ill-understood. Not all of those infected individuals will develop acquired immunodeficiency syndrome (AIDS). Indeed recent reports have suggested that there may be certain individuals that are resistant to HIV-1 infection.

C-C chemokine receptor (CCR)-5 is the principal co-receptor of the macrophage (Mϕ)-tropic human immunodeficiency virus (HIV)-1 (Cocchi *et al., Science,* 270:1811-1815, 1995; Deng *et al., Nature*, 381:661-666, 1996; Dragic *et al., Nature,* 381:667-673, 1996; Alkhatib *et al., Science,* 272:1955-1958, 1996; Choe *et al., Cell,* 85:1135-1148, 1996; Doranz *et al., Cell,* 85:1149-1158, 1996). Several recent studies have shown that individuals with a homozygous defect in CCR5 are resistant to HIV-1 infection with no apparent clinical conditions associated with the CCR5 defect. HIV-1 infected individuals with a heterozygous CCR5 defect exhibit slower disease progression (Liu *et al., Cell,* 86:367-377, 1996; Samson *et al., Nature,* 382:722-725, 1996; Dean *et al., Science,* 273:1856-1862, 1996). In addition, some individuals whose lymphocytes express high levels of CC-chemokines are partially resistant to HIV-1 infection (Paxton *et al., Nature Med.*, 2:412-417, 1996).

In light of the foregoing data it would be therapeutically beneficial to prevent HIV-1 binding to the C-C receptors and thereby prevent HIV-1 infection of cells. One way to achieve this would be to prevent cell surface expression of the receptor. There are numerous methods of attempting abrogation of expression of a protein. These include homologous recombination, antisense and ribozyme technologies and single chain antibodies to bind the proteins intracellularly. Each of these techniques has particular disadvantages. Homologous recombination techniques are difficult to use in a clinical applications, whereas anitsense and ribozyme technologies require introduction of high concentrations of genetic material and present incomplete and non-specific effects. The monoclonal antibody approach takes a long time to develop and has the further complication that the antibody may be too specific and thus will not achieve a broad enough inhibition.

Thus it is clear that an easy efficient technique is needed to prevent the C-C chemokine receptors from being expressed at the cell surface of lymphocytes so that they do not present the opportunity for HIV to infect the cells.

SUMMARY OF THE INVENTION

The present invention seeks to overcome certain drawbacks in the prior art by providing compositions and methods for use in therapy or prevention of HIV infection and in the prevention or treatment of opportunistic infections in AIDS or ARC patients. The present invention provides lymphocytes that are resistant to HIV infection due to the lack of an expressed co-receptor. By co-receptor is meant a receptor on the lymphocyte surface that is necessary for HIV infection, other than the CD4 receptor, which is also necessary for HIV-1 infection. In an embodiment of the invention, healthy lymphocytes that are resistant to HIV infection may be provided to a patient, thus maintaining a desirable level of immune cells during an HIV infection, thus helping the patient resist secondary infections. The compositions and methods disclosed herein will be particularly effective in conjunction with other forms of therapy, such as AZT and/or protease inhibitors that are designed to inhibit viral replication, by maintaining desirable levels of white blood cells. This, in effect, buys the patient the time necessary for the anti-viral therapies to work.

The present invention may be described in a certain broad aspect as an expression vector, wherein the expression region comprises in a 5' to 3' orientation: a promoter; an intracellular

retention signal sequence encoding region; and a chemokine encoding gene; wherein the intracellular retention signal sequence and the chemokine encoding gene are expressed as a single intrakine transcript. The term "intrakine" has been coined by the present inventors to indicate a chemokine that is directed by a signal sequence to be retained in an intracellular compartment such as the lumen of the endoplasmic reticulum, the Golgi apparatus, a lysosome, an intracellular vesicle or other intracellular organelle. A vector of the present invention may also encode a secreted chemokine, preferably expressed from an internal ribosome entry site (IRES). The secreted chemokine preferably binds to the same chemokine receptor as the expressed intrakine. In this way, the transduced cells that are resistant to HIV infection because of phenotypic knockout of the co-receptor are also able to inhibit infection of non-transduced, susceptible cells, by secreting a chemokine that competes with HIV for co-receptor binding. The secreted chemokine and/or the intrakine may also be a mutated form of chemokine that maintains receptor binding, but lacks biological activity. Such a chemokine analog in which eight amino acids are deleted from the N-terminus is described by Arenzana-~~Seladeedos~~ Seisdedos *et al*, *Nature*, 383: 400, 1996 (incorporated herein by reference). In the practice of the invention, one or more of the N-terminal amino acids may be deleted to obtain such a chemokine analog.

A preferred expression vector of the present invention is a retroviral vector, but any type of vector known in the art may be used. For example, one may use an adenoviral vector, an adeno associated viral vector, a plasmid, a cosmid, liposome encapsulated DNA or even RNA in the practice of the invention. The inventors have demonstrated herein the use of an endoplasmic reticulum retention signal sequence, however, any signal sequence that directs a translation product to an intracellular organelle as described above may be used. A preferred signal sequence is a KDEL sequence. (SEQ ID NO: 4)

The expression vector of the present invention preferably encodes a chemokine gene product that binds to a C-C chemokine 5 receptor, a C-C chemokine 3 receptor, a C-C chemokine 1 receptor or a CXR4 receptor. Preferred chemokines include, but are not limited to RANTES, MIP-1α or SDF.

The present invention may also be described in certain broad aspects as a method of inhibiting phenotypic expression of a chemokine receptor in a cell, wherein the method comprises blocking cell surface expression of a chemokine receptor. This method may be further defined as comprising the steps of: obtaining a vector comprising a nucleic acid segment encoding in a 5' to 3' orientation, a promoter, an intracellular retention signal sequence and a

chemokine receptor binding polypeptide gene; and transducing the vector into the cell; wherein the vector expresses the nucleic acid sequence to produce a fusion polypeptide when transduced into the cell. The expressed polypeptide may be a chemokine, a chemokine analog, such as a chemokine with an N-terminal deletion of up to eight amino acids, an antibody such as a single chain antibody or a peptide that binds the receptor. In the practice of the invention, one may, using techniques well known in the art, isolate peptides from a peptide expression library, for example, that are able to bind the chemokine receptors described herein. The expression of any of such peptides as a fusion with a leader sequence to direct the peptide/receptor to an intracellular organelle, as described herein, either a known or newly discovered peptide would be encompassed by the present invention. Preferred polypeptides include RANTES, MIP-1α, SDF, HIV gp120 or the V3 region of HIV gp120.

In certain broad aspects, the invention may be described as a method of inhibiting HIV infection of a cell comprising phenotypic knock-out of an HIV co-receptor in the cell. In the practice of this method, the co-receptor is preferably the C-C chemokine 5 receptor, the C-C chemokine 3 receptor, the C-C chemokine 1 receptor or the CXR4 receptor. The phenotypic knockout may be by any method known in the art, such as anti-sense expression, genomic recombination, or preferably by expressing a receptor binding polypeptide fused to an intracellular retention signal sequence in the cell. In the practice of the method, an intracellular retention signal sequence directs the fusion polypeptide to the endoplasmic reticulum, the Golgi apparatus, a lysosome or intracellular vesicle or other intracellular organelle. In the practice of such methods, the vector may be a viral vector, or even a retroviral vector, for example, and the cell may be a lymphocyte, monocyte, macrophage or a stem cell. A preferred intracellular retention signal sequence is an endoplasmic reticulum signal sequence such as a KDEL sequence (SEQ ID NO: 7). The receptor binding polypeptide of the present method is preferably a CC-chemokine, a CXC chemokine, an analog of a CC or CXC chemokine, a single chain antibody, an HIV gp120 protein, a V3 region of HIV gp120 or a peptide that binds to the receptor.

In an exemplary embodiment of the method, a cell is transduced with a CC-chemokine gene fused to an endoplasmic reticulum (ER)-retention signal to intracellularly block the transport and surface expression of an endogenous CC receptor, especially a CCR5, CCR3 or CCR1 receptor. In an alternate exemplary embodiment, a cell is transduced with a CXC-chemokine gene fused to an endoplasmic reticulum (ER)-retention signal to intracellularly block the transport and surface expression of an endogenous CXR4 receptor.

Another broad aspect of the present invention is a method of treating an HIV infection in a subject comprising administering to the subject lymphocytes, monocytes, macrophages or stem cells wherein the administered cells exhibit a phenotypic knock out of an HIV co-receptor. In a preferred embodiment of the method, the cells are transduced *ex vivo* with a vector that expresses a polypeptide that is able to bind the newly expressed vector and retain it in an intracellular organelle as described herein. The cells may preferably be autologous lymphocytes, macrophages, monocytes, stem cells or even heterologous stem cells. In an alternate embodiment, the cell may express an antisense RNA effective to block expression of the co-receptor.

An embodiment of the present invention is also a method of increasing or maintaining a white blood cell (WBC) count in a subject with an HIV infection comprising administering to the subject a pharmaceutical composition comprising lymphocytes, monocytes, macrophages or stem cells transduced with a vector of the invention as described herein. In the practice of the method, the WBC count may be monitored on a regular basis, and when the count drops below a certain critical or dangerous level, then the transduced cells of the invention would be administered in an amount effective to keep the WBC count above the desired level. The cells would be re-administered every few weeks to months as needed. The intravenous infusion of cells in a pharmaceutical composition is well known in the art and could be practiced by the skilled practitioner in light of the present disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Schematic diagram of the strategy to phenotypically knock-out CCR5. Lymphocytes or stem cells are genetically modified to co-express a mutated chemokine, targeted to the lumen of the endoplasmic reticulum (ER) that binds intracellularly and prevents the transport and surface expression of newly synthesized CCR5. The cells also co-express a native chemokine to be secreted out of the cell to competitively inhibit HIV-1 infection of susceptible cells. As a result, the transduced cells with the phenotypic CCR5 knock out are not only

resistant to HIV-1 infection, but also produce chemokines to extracellularly inhibit HIV-1 infection in susceptible cells.

FIG. 2. Schematic representation of construction of expression vectors. The human MIP-1α and RANTES cDNA genes and their derivatives containing an ER retention signal (KDEL) (SEQ ID NO:7) were cloned into the pRc/CMV vector (Invitrogen). Bi-cistronic vectors for co-expression of the native and mutated chemokines using an internal ribosome-entry site (IRES) sequence (Chen *et al.*, *Human Gene Ther.*, 7:1515-1526, 1996) were constructed, and further cloned into a retroviral vector (pLNCX) (Miller, *Curr. Top. Microbiol. Immunol.*, 158:1-24, 1992). A chimeric construct containing a short influenza hemagglutinin (HA) tag sequence (YPYDVPDYA SEQ ID NO:1) (Field *et al.*, *Mol. Cell. Biol.*, 8:2159-2165, 1988) fused to the N-terminus of CCR5 (Liu *et al.*, 1996) was also generated. Ψ, packaging sequence.

FIG. 3A, FIG. 3B, FIG. 3C and FIG. 3D. Blockade of surface expression of CCR5 by the ER-retained MIP1-K. COS cells on 6-well plates were transfected with 2.5 μg of pCMV-HA-CCR5 alone (FIG. 3B), or co-transfected with different amounts of pCMV-MIP1-K (FIG. 3C and FIG. 3D). 48 hr later, the suspensions of transfected cells were incubated with an anti-HA antibody (BAbCo, Richmond, CA), and then stained with an anti-rabbit IgG-FITC conjugate (Sigma). COS cells were directly incubated with the second antibody conjugate as a negative control (FIG. 3A).

FIG. 3E. Inhibition of CCR5-mediated syncytium formation. HeLa-T4$^+$ cells grown on 6-well plates were transfected with 2 μg of pCMV-CCR5 alone, or co-transfected with different amounts of chemokine expression vectors. 48 hr later, the transfected cells were co-cultured with COS cells expressing Mϕ-tropic (ADA or YU2), or T-tropic envelope proteins (IIIB), and the syncytia in each well (duplicate) were counted 12 to 24 hr later. The percentages of inhibition of syncytium formation are presented. The syncytium formation of ADA and YU2 was effectively inhibited by MIP1-K and RANTES-K intrakines, but the T-tropic envelope-mediated syncytium formation was not inhibited by these intrakines.

FIG. 4A. Schematic representation of construction of expression vectors. The SDF-1 gene from a mouse spleen cDNA library was fused to a sequence encoding an ER retention signal (KDEL) (SEQ ID NO:7) and cloned into the pRc/CMV vector (Invitrogen). Bi-cistronic vectors for co-expression of the native and mutated chemokine using an internal ribosome-entry site (IRES) sequence (Chen *et al.*, 1996) were constructed, and further cloned into a retroviral vector (pLNCX) (Miller, 1992). A chimeric construct containing a short influenza hemagglutinin (HA)

tag sequence (YPYDVPDYA SEQ ID NO:1) (Field *et al.*, 1988) fused to the N-terminus of fusin (CXCR4) (Liu *et al.*, 1996) was also generated. Ψ, packaging sequence.

FIG. 4B. Resistance to T-tropic HIV-1 infection of transduced lymphocytes when transduced with ER targeted SDF. Diamonds, JK-CTRL; Squares, JK-SDF-KDEL; Triangles, JK-SDF.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention provides methods for treating and preventing HIV-1 infection. In particular, the present invention employs novel methods for preventing the cell surface expression of C-C chemokine receptors, thereby preventing the binding of HIV to its target cells. The present invention also provides novel compositions for use in the treatment and management of HIV infection.

The phenotypic CCR knock-out can be achieved by genetically modifying lymphocytes to express intrakines, which result in resistance of permissive cells to Mϕ-tropic HIV-1 infection. Significant advantages of this novel anti-HIV approach are outlined as follows: Given the well-documented importance of CCR5 in HIV-1 infection and disease progression, and the lack of adverse clinical conditions associated with defects of CCR5 expression, reinfusion of genetically modified lymphocytes or stem cells with phenotypic CCR5 knock-out may enable an individual to partially or completely resist HIV-1 infection, and prevent or delay disease progression.

Currently described anti-HIV approaches are primarily targeted at viral components, such as viral envelope proteins, Tat, Rev or reverse transcriptase (RT), by anti-sense constructs, ribozymes, dominant negative mutants, intrabodies, or ER-retained CD4. A major problem facing anti-HIV therapy is frequent viral mutation, resulting in viral resistance. In contrast, the novel anti-HIV approach described herein is uniquely targeted at the cellular receptor. As a result, frequent HIV mutations may be unable to evade this strategy. It should be noted that the CD4 receptor is functionally indispensable, and therefore, not an appropriate target for this strategy.

Intrakines not only block CCR5, but also CCR3 and CCR1 which were also reported as co-receptors for some HIV-1 viruses, suggesting a broad effect on HIV-1. In addition, phenotypic CCR knock-out combined with extra-cellular inhibition of susceptible cells by

secreted chemokines is contemplated to have a synergistic anti-HIV effect. A further advantage of the present invention is that this approach is less technically challenging than other gene therapy approaches. CCR5 expression in human lymphocytes is very low (*i.e.*, it cannot be detected in radiolabeling), and therefore, expression levels of intrakines achievable by currently used expression vectors are expected to be sufficient to inactivate CCR5.

Another potential problem facing gene therapy is the host immune response (cytotoxic T-cells) that destroys genetically modified cells expressing foreign proteins. However, transduced cells expressing human chemokines as in the present disclosure would not generate new antigens. In addition, this approach can be used to phenotypically knock-out other receptors such as T-tropic HIV-1 co-receptor (Feng *et al.*, *Science,* 272:872-877, 1996). Thus, this novel strategyas described herein provides an effective gene therapy for HIV-1 infection.

1. Human Immunodeficiency Virus

HIV is classified as a retrovirus because it contains reverse transcriptase. Infection of cells with HIV usually results in cell death. HIV presents two major antigenic types, HIV-1 and HIV-2, that are readily distinguishable by differences in antibody reactivity to the envelope glycoprotein. HIV-1 and HIV-2 share about 40% homology. It has been reported that HIV-1 is more efficient at causing AIDS than HIV-2.

The first step of HIV infection is the high affinity binding of gp 120 glycoprotein to the CD4 receptor, present on the surface of many cell types including T4 cells, monocyte-macrophages, dendriditic cells and cells of the central nervous system. The high affinity of the HIV envelope glycoprotein for the CD4 receptor is a crucial step in the pathogenesis of HIV since the major cells which express CD4 are the target cells (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Maddon *et al.*, 1986). Because T4 lymphocyte cells play a pivotal role in all aspects of the immune system, death or impairment of T4 lymphocyte function results in catastrophic immune dysfunction.

There are several ways in which HIV infection may directly result in the destruction of T4 cell function. HIV replication may kill T4 cells as a result of destruction of the cell membrane by viral proteins. Alternatively the production of large amounts of viral genetic material and proteins may interfere with normal cell metabolism and finally, HIV may also infect and destroy progenitor cells that are responsible for the propagation of the lymphoid cell pool.

HIV infection may also indirectly cause T4 cell death. In one such mechanism it is thought that an autoimmune phenomena is triggered in which anti-HIV immune responses are targeted at uninfected T4 cells that either have free envelope proteins bound to their membrane or present processed antigens. Additionally since both the HIV envelope protein and the class II major histocompatibility complex (MHC) antigens bind to the CD4 receptor, their common binding sites may represent cross reacting antigens. Thus anti-HIV antibodies may react with uninfected T4 cells that express class II MHC molecules. Also it may be that the anti-HIV-immune effector cells kill many infected cells.

The monocyte-macrophage is a target for HIV infection both *in vivo* and *in vitro*. Infection may occur through the CD4 receptor or via phagocytosis. Unlike T4 cells, monocyte macrophages appear to be resistant to cell lysis. The virus is able to replicate intracellularly in monocyte macrophages with virions budding into intracytoplasmic vesicles. As a result, viral antigens may not be expressed at the cell surface thereby enabling the monocyte-macrophage to escape immune surveillance and to transmit infection to other organs.

There are a broad range of immune responses against HIV at all stages of infection. Antibodies produced throughout the course of the HIV infection and subsequent AIDS manifestation seem ineffective at halting the progress of this persistent infective disorder. The expression of genetic variants of HIV *in vivo* during the progress of the disease is likely to be one way in which HIV evades humoral and cellular immune responses.

Most primary HIV-1 viruses that initiate human infection and persist throughout the course of infection replicate to low levels in peripheral blood mononuclear cells but do not replicate in immortalized T cell lines (Schuitemaker *et al.*, 1991; 1992 Conner *et al.*, 1993; Conner and ho, 1994a and 1994b). These viruses are referred to herein as macrophage tropic primary isolates. In some HIV-1 infected individuals viruses that replicate to higher levels in PMBC and that can infect and induce the formation of syncytia in immortalized CD4 cell lines emerge late in the course of infection (Schuitemaker *et al.*, 1992 Conner *et al.*, 1993; Conner and Ho, 1994a and 1994b). These are referred to as T cell line-tropic primary viruses.

2. Chemokine Receptors

There is a receptor that has been variously termed HUMSTSR, LCR-1, or LESTR and has been shown to allow a range of non-human, CD4-expressing cells to support infection and cell fusion. It has also been termed "fusin". This receptor is also referred to herein as CXR4.

Antibodies against HUMSTSR have been shown to block fusion and infection by laboratory adapted HIV-1 isolates but not by macrophage tropic primary viruses.

It has further been observed that infection by macrophage tropic primary isolates but not laboratory adapted isolates can be inhibited by the β chemokines RANTES, MIP-1α and MIP-1β (Cocchi *et al.*, 1995). A high endogenous expression of β-chemokines has been suggested to account for the *in vitro* resistance to HIV-1 infection of CD4+ T cells from uninfected individuals who have been exposed to seropositive partners (Paxton *et al.*, 1996). This resistance was only seen from macrophage tropic and not T cell-line tropic viruses and was influenced by the structure of gp 120. It was suggested that at least one other host cell surface molecule besides CD4 and distinct from HUMSTSR facilitates entry of the primary macrophage tropic HIV isolates and that this factor might be influenced by interaction with the β-chemokines.

G-protein coupled receptors respond to a variety of chemoattractants, neurotransmitters, hormones and the like. Seven transmembrane receptors that transduce their signals through heterotrimeric G proteins are used by leukocytes to respond to chemokines (Horuk, 1994). Chemokines are a family of structurally related peptides that recruit leukocytes to inflammatory lesions, induce the reels of granule contents from granulocytes, regulate integrin avidity and exhibit proinflammatory properties.

The α chemokines or CXC chemokines act upon neutrophils whilst the β-chemokines, or CC-chemokines, act upon monocytes, lymphocytes, basophils and eosinophils (Baggiolini *et al.*, 1994; Schall and Bacon, 1994). Thus the CC chemokine receptors potentially exhibit a tissue distribution consistent with the known tropism of HIV-1. There are a number of closely related CC chemokine receptors, five of which have been characterized by ligand binding assays. These are designated CCR1, CCR2A, CCR2B, CCR3, CCR4 and CCR5.

CCR-5 is a seven-transmembrane glycoprotein that is synthesized at the ER and transported to the plasma membrane through the secretory pathway (Samson *et al.*, *Biochemistry*,. 35:3362-3367, 1996; Strader *et al.*, 1994). CXR4 is a seven-transmembrane glycoprotein that is synthesized at the ER and transported to the plasma membrane, where CXR4 binds its ligand or the HIV-1 envelope protein with high affinity (Strader *et al.*, 1994).

3. Intrakine Polypeptides

The chemokines, RANTES, MIP-1α, and MIP-1β which are produced by macrophages, lymphocytes and other cells, and bind their receptors (CCR-5, -3 and -1) with high affinity

(Neote *et al., Cell,* 72:415-425, 1993; Schall *et al., J. Immunol.,* 141:1018-1025, 1988; Gong *et al., J. Biol. Chem.,* 271:10521-10527, 1996), have been shown to suppress HIV-1 infection, particularly in macrophage tropic isolates. It is believed that the suppressive C-C chemokines exert their infection inhibiting activities by binding to a chemokine receptor for macrophage tropic HIV-1 isolates thus inhibiting fusion mediated by the corresponding env glycoproteins.

Thus RANTES MIP-1α and MIP-1β are potent inhibitors of macrophage tropic infection *in vitro*. They are produced in elevated levels by CD8+ cells from HIV-1 infected individuals and by CD4+ cells obtained from long term, high risk seronegative individuals and are thought to be refractory to HIV-1 infection *ex vivo*. SDF-1 is a member of the CXC-chemokines, and is constitutively expressed by bone-marrow-derived stromal cells (Nagasawa, *PNAS*, 1994; Tashiro, *Science*, 1993). SDF-1 was recently identified as a biological ligand of fusin/CXR4, which is a co-receptor for T-tropic HIV-1 virus (Neote *et al.*, 1993; Schall *et al.*, 1988; Gong *et al.*, 1996).

Blockade of cell surface expression of a membrane protein by intracellular binding of intrabodies or other molecules targeted to the ER has been demonstrated in the inventors' and other studies (Marasco *et al., Proc. Natl. Acad. Sci. USA,* 90:7889-7893, 1993; Chen *et al., Exp. Opin. Invest. Drugs,* 4:823-833, 1995a; Chen *et al., Human Gene Therapy*, 5:595-601, 1995b; Buonocore and Rose, *Nature,* 345:625-628, 1990. The chemokines RANTES, MIP-1α and SDF-1 were used by the inventors to block the cell surface expression of C-C chemokine receptors.

The present invention contemplates altering the chemokine receptor ligands so that the ligands may be targeted to the endoplasmic reticulum. These molecules are herein termed intrakines. By "intrakine" is meant any ligand that binds to a C-C chemokine receptor at the cell surface but has been modified to be targeted to the ER of the lymphocyte or other intracellular organelle, such ligands include but are not limited to RANTES, MIP-1α, MIP-1β, for binding to CCR5 and stromal cell derived factor-1(SDF-1) for binding to CCR4.

4. Intrakine-Encoding Polynucleotides

The polynucleotides according to the present invention may encode an entire intrakine gene, a functional intrakine protein domain, or any intrakine polypeptide. "Complementary" polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller

pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

As used herein, the term "complementary sequences" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have a complementary nucleotide at thirteen or fourteen positions. Naturally, sequences which are "completely complementary" will be sequences which are entirely complementary throughout their entire length and have no base mismatches.

Other sequences with lower degrees of homology also are contemplated. For example, an genetic construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, a ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

The polynucleotides may be derived from genomic DNA, *i.e.*, cloned directly from the genome of a particular organism. In other embodiments, however, the polynucleotides may be complementary DNA (cDNA). cDNA is DNA prepared using messenger RNA (mRNA) as template. Thus, a cDNA does not contain any interrupted coding sequences and usually contains almost exclusively the coding region(s) for the corresponding protein. In other embodiments, the polynucleotide may be produced synthetically.

It may be advantageous to combine portions of the genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. Introns may be derived from other genes in addition to intrakine. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

It is contemplated that natural variants of intrakine exist that have different sequences than those disclosed herein. Thus, the present invention is not limited to use of any polynucleotide sequence for Intrakine but, rather, includes use of any naturally-occurring variants. The present invention also encompasses chemically synthesized mutants of these sequences.

Another kind of sequence variant results from codon variation. Because there are several codons for most of the 20 normal amino acids, many different DNA's can encode the Intrakine. Reference to the following table will allow such variants to be identified.

Table 1

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

Allowing for the degeneracy of the genetic code, sequences that have between about 50% and about 75%, or between about 76% and about 99% of nucleotides that are identical to the nucleotides of the known chemokine genes will be preferred. Sequences that are within the scope of "an intrakine-encoding polynucleotide" are those that are capable of base-pairing with a polynucleotide segment set forth above under intracellular conditions.

It also is well understood by the skilled artisan that, inherent in the definition of a biologically functional equivalent protein or peptide, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent peptides are thus defined herein as those peptides in which certain, not most or all, of the amino acids may be substituted. In particular, where the N-terminus of the protein is concerned, it is contemplated that only about 16 or more preferably, about 5 amino acids may be changed within a given peptide. Of course, a plurality of distinct proteins/peptides with different substitutions may easily be made and used in accordance with the invention.

Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. An analysis of the size, shape and type of the amino acid side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all a similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as biologically functional equivalents.

In making changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte & Doolittle, 1982, incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent protein. As detailed in U.S. Patent

4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

Site-specific mutagenesis. Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art. As will be appreciated, the technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector which includes within its sequence a DNA sequence encoding the desired protein. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a

heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants of genes may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

5. Knockout Strategies

In light of the foregoing discussion, the critical importance of CCR5 for HIV-1 infection and disease progression, and the dispensable nature of CCR5 suggest that the knock-out of CCR5 in stem cells or lymphocytes may have a therapeutic implication. The present invention may be used in conjunction with a variety of methods in order to reduce, abrogate or knockout the expression of the CCR5 receptor at the cell surface of the lymphocyte or other cell at which the CCR5 receptor may be expressed. These methods are described herein below.

ER targeting of Intrakines. In preferred embodiments of the present invention, phenotypic CCR5 knock-out is accomplished by transducing cells with a mutated CC-chemokine gene containing an endoplasmic reticulum (ER)-retention signal to intracellularly block the transport and surface expression of the newly synthesized CCR5. Human peripheral blood lymphocytes (PBLs) expressing the intracellular chemokine, termed "intrakine," were found to resist Mϕ-tropic HIV-1 infection. Furthermore, secreted chemokines as well as intrakines were co-expressed for additional inhibition of viral infection. Thus, this novel anti-HIV approach uniquely targeted at the cellular receptors, rather than the viral components used by other anti-HIV approaches, may overcome frequent mutations of HIV-1, and, therefore, should have significant implications for gene-based treatment and even prevention of HIV-infection.

The tubular architecture of the ER and the directional flow of proteins through the secretory system combine to make phenotypic knockout of a receptor at the ER level an extremely effective mechanism of inhibition of cell surface receptor expression. Molecules intended for localization and targeting to the ER are generally equipped with a leader peptide and a C-terminus ER retention signal. One such signal is a KDEL amino acid motif (Munro and

(SEQ ID NO: 4)

Pelham, *Cell,* 48:899-907, 1987). Thus the present invention employs genetic recombination techniques described herein to engineer an ER retention signal to chemokines thereby targeting these ligands to the ER where they bind the CCR proteins thereby preventing their expression at the cell surface.

Antisense. Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either in vitro or in vivo, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches.

For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

Ribozymes. Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cook, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cook *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cook *et al.*, 1981). For example, U.S. Patent No. 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-ras, c-fos and genes of HIV.

Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

Homologous Recombination. Another approach for preventing CCR5 expression at the cell surface involves the use of homologous recombination, or "knock-out technology". Homologous recombination relies, like antisense, on the tendency of nucleic acids to base pair with complementary sequences. In this instance, the base pairing serves to facilitate the interaction of two separate nucleic acid molecules so that strand breakage and repair can take place. In other words, the "homologous" aspect of the method relies on sequence homology to bring two complementary sequences into close proximity, while the "recombination" aspect provides for one complementary sequence to replace the other by virtue of the breaking of certain bonds and the formation of others.

Put into practice, homologous recombination is used as follows. First, the target gene is selected within the host cell, in this case, CCR5. Sequences homologous to the CCR5 target gene are then included in a genetic construct, along with some mutation that will render the target gene inactive (stop codon, interruption, and the like). The homologous sequences flanking the inactivating mutation are said to "flank" the mutation. Flanking, in this context, simply means that target homologous sequences are located both upstream (5') and downstream (3') of the mutation. These sequences should correspond to some sequences upstream and downstream of the target gene. The construct is then introduced into the cell, thus permitting recombination between the cellular sequences and the construct.

As a practical matter, the genetic construct will normally act as far more than a vehicle to interrupt the gene. For example, it is important to be able to select for recombinants and, therefore, it is common to include within the construct a selectable marker gene. This gene permits selection of cells that have integrated the construct into their genomic DNA by conferring resistance to various biostatic and biocidal drugs. In addition, a heterologous gene that is to be expressed in the cell also may advantageously be included within the construct. The arrangement might be as follows:

...vector•5'-flanking sequence•heterologous gene•selectable marker gene•flanking sequence-3'•vector...

Thus, using this kind of construct, it is possible, in a single recombinatorial event, to (i) "knock out" an endogenous gene, (ii) provide a selectable marker for identifying such an event and (iii) introduce a heterologous gene for expression.

Another refinement of the homologous recombination approach involves the use of a "negative" selectable marker. This marker, unlike the selectable marker, causes death of cells which express the marker. Thus, it is used to identify undesirable recombination events. When seeking to select homologous recombinants using a selectable marker, it is difficult in the initial screening step to identify proper homologous recombinants from recombinants generated from random, non-sequence specific events. These recombinants also may contain the selectable marker gene and may express the heterologous protein of interest, but will, in all likelihood, not have the desired "knock out" phenotype. By attaching a negative selectable marker to the construct, but outside of the flanking regions, one can select against many random recombination events that will incorporate the negative selectable marker. Homologous recombination should not introduce the negative selectable marker, as it is outside of the flanking sequences.

Single Chain Monoclonal Antibodies. Single chain antibodies, synthesized by the cell and targeted to a particular cellular compartment can be used to interfere in a highly specific manner with cell growth and metabolism. Recent application include the phenotypic knockout of growth factor receptors, the functional inactivation of p21 and the inhibition of HIV-1 replication.

Methods for the production of single-chain antibodies are well known to those of skill in the art. The skilled artisan is referred to US Patent Number 5,359,046, (incorporated herein by reference) for such methods. A single chain antibody is created by fusing together the variable domains of the heavy and light chains using a short peptide linker, thereby reconstituting an antigen binding site on a single molecule.

Single-chain antibody variable fragments (Fvs) in which the C-terminus of one variable domain is tethered to the N-terminus of the other via a 15 to 25 amino acid peptide or linker, have been developed without significantly disrupting antigen binding or specificity of the binding (Bedzyk *et al.*, 1990; Chaudhary *et al.*, 1990). These Fvs lack the constant regions (Fc) present in the heavy and light chains of the native antibody.

In principle, the high affinity and selective binding properties of intracellular antibodies or intrabodies can be used to modulate cellular physiology and metabolism by a wide variety of mechanisms. For example binding of an intrabody may be used to block or stabilize macromolecular interactions, modulate enzyme function by occluding an active site, sequestering substrate or fixing the enzyme in an active or an inactive conformation as the need may be. Intrabodies may also be used to divert proteins from their usual cellular compartment for

example by sequestering transcription factors in the cytoplasm, or by retention in the ER of the proteins destined for the cell surface. In this regard intrabodies may be useful in conjunction with the present invention to prevent CCR5 expression at the cell surface and thereby inhibit HIV-1 infectivity.

5

6. Expression Vectors

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. Thus, in certain embodiments, expression includes both transcription of an intrakine gene and translation of an intrakine mRNA into an intrakine protein product. In other embodiments, expression only includes transcription of the nucleic acid encoding an intrakine or its complement.

In order for the construct to effect expression of at least an intrakine transcript, the polynucleotide encoding the Intrakine polynucleotide will be under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the host cell, or introduced synthetic machinery, that is required to initiate the specific transcription of a gene. The phrase "under transcriptional control" or "operatively linekd" means that the promoter is in the correct location in relation to the polynucleotide to control RNA polymerase initiation and expression of the polynucleotide.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a Intrakine polynucleotide is not believed to be critical, so long as it is capable of expressing the polynucleotide in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the polynucleotide coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of the Intrakine polynucleotide. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of polynucleotides is contemplated as well, provided that the levels of expression are sufficient to produce a growth inhibitory effect.

By employing a promoter with well-known properties, the level and pattern of expression of a polynucleotide following transfection can be optimized. For example, selection of a promoter which is active in specific cells, such as tyrosinase (melanoma), alpha-fetoprotein and albumin (liver tumors), CC10 (lung tumor) and prostate-specific antigen (prostate tumor) will permit tissue-specific expression Intrakine polynucleotides. Table 2 lists several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of Intrakine constructs. This list is not intended to be exhaustive of all the possible elements involved in the promotion of Intrakine expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation.

Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of an intrakine construct. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacteriophage promoters if the appropriate bacteriophage polymerase is provided, either as part of the delivery complex or as an additional genetic expression vector.

Table 2

ENHANCER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ ß
ß-Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DRα
ß-Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase *I*
Metallothionein
Collagenase
Albumin Gene
α-Fetoprotein
τ-Globin
ß-Globin
c-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α_1-Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)

Table 2 (Continued)

Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the Intrakine construct. For example, with the polynucleotide under the control of the human PAI-1 promoter, expression is inducible by tumor necrosis factor. Table 3 illustrates several promoter/inducer combinations:

Table 3

Element	Inducer
MT II	Phorbol Ester (TFA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
ß-Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H_2O_2
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone

In certain embodiments of the invention, the delivery of an expression vector in a cell may be identified *in vitro* or *in vivo* by including a marker in the expression vector. The marker would result in an identifiable change to the transfected cell permitting easy identification of expression. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants. Alternatively, enzymes such as herpes simplex virus thymidine kinase (tk) (eukaryotic) or chloramphenicol acetyltransferase (CAT) (prokaryotic) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed along with the polynucleotide encoding Intrakine. Further examples of selectable markers are well known to one of skill in the art.

One will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Also contemplated as an element of the expression construct is a terminator. These elements can serve to enhance message levels and to minimize read through from the construct into other sequences.

In some embodiments of the present invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis and, in some cases, integrate into the host cell chromosomes, have made them attractive candidates for gene transfer in to mammalian cells. However, direct uptake of naked DNA, as well as receptor-mediated uptake of DNA complexes have been demonstrated, expression vectors need not be viral but, instead, may be any plasmid, cosmid or phage construct that is capable of supporting expression of encoded genes in mammalian cells, such as pUC or Bluescript™ plasmid series.

Retroviruses. The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes - *gag*, *pol*, and *env* - that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the *gag* gene, termed Ψ, functions as a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5′ and 3′ ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding Intrakine is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the *gag*, *pol* and *env* genes but without the LTR and Ψ components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a human cDNA, together with the retroviral LTR and Ψ sequences is introduced into this cell line (by calcium phosphate precipitation for example), the Ψ sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin,

1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes via sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

Adenoviruses. Human adenoviruses are double-stranded DNA tumor viruses with genome sizes of approximate 36 kb (Tooze, 1981). As a model system for eukaryotic gene expression, adenoviruses have been widely studied and well characterized, which makes them an attractive system for development of adenovirus as a gene transfer system. This group of viruses is easy to grow and manipulate, and they exhibit a broad host range *in vitro* and *in vivo*. In lytically infected cells, adenoviruses are capable of shutting off host protein synthesis, directing cellular machineries to synthesize large quantities of viral proteins, and producing copious amounts of virus.

The E1 region of the genome includes E1A and E1B which encode proteins responsible for transcription regulation of the viral genome, as well as a few cellular genes. E2 expression, including E2A and E2B, allows synthesis of viral replicative functions, *e.g.* DNA-binding protein, DNA polymerase, and a terminal protein that primes replication. E3 gene products prevent cytolysis by cytotoxic T cells and tumor necrosis factor and appear to be important for viral propagation. Functions associated with the E4 proteins include DNA replication, late gene expression, and host cell shutoff. The late gene products include most of the virion capsid proteins, and these are expressed only after most of the processing of a single primary transcript from the major late promoter has occurred. The major late promoter (MLP) exhibits high efficiency during the late phase of the infection (Stratford-Perricaudet and Perricaudet, 1991a).

As only a small portion of the viral genome appears to be required *in cis* (Tooze, 1981), adenovirus-derived vectors offer excellent potential for the substitution of large DNA fragments when used in connection with cell lines such as 293 cells. Ad5-transformed human embryonic kidney cell lines (Graham, *et al.*, 1977) have been developed to provide the essential viral proteins *in trans*.

Particular advantages of an adenovirus system for delivering foreign proteins to a cell include (i) the ability to substitute relatively large pieces of viral DNA by foreign DNA; (ii) the structural stability of recombinant adenoviruses; (iii) the safety of adenoviral administration to humans; and (iv) lack of any known association of adenoviral infection with cancer or malignancies; (v) the ability to obtain high titers of the recombinant virus; and (vi) the high infectivity of Adenovirus.

In general, adenovirus gene transfer systems are based upon recombinant, engineered adenovirus which is rendered replication-incompetent by deletion of a portion of its genome, such as E1, and yet still retains its competency for infection. Sequences encoding relatively large foreign proteins can be expressed when additional deletions are made in the adenovirus genome. For example, adenoviruses deleted in both E1 and E3 regions are capable of carrying up to 10 Kb of foreign DNA and can be grown to high titers in 293 cells (Stratford-Perricaudet and Perricaudet, 1991a). Surprisingly persistent expression of transgenes following adenoviral infection has also been reported.

Adenovirus-mediated gene transfer has recently been investigated as a means of mediating gene transfer into eukaryotic cells and into whole animals. For example, in treating mice with the rare recessive genetic disorder ornithine transcarbamylase (OTC) deficiency, it was found that adenoviral constructs could be employed to supply the normal OTC enzyme. Unfortunately, the expression of normal levels of OTC was only achieved in 4 out of 17 instances (Stratford-Perricaudet *et al.*, 1991b). Therefore, the defect was only partially corrected in most of the mice and led to no physiological or phenotypic change.

Attempts to use adenovirus to transfer the gene for cystic fibrosis transmembrane conductance regulator (CFTR) into the pulmonary epithelium of cotton rats have also been partially successful, although it has not been possible to assess the biological activity of the transferred gene in the epithelium of the animals (Rosenfeld *et al.*, 1992). Again, these studies demonstrated gene transfer and expression of the CFTR protein in lung airway cells but showed no physiologic effect. In the 1991 *Science* article, Rosenfeld *et al.* showed lung expression of

a1-antitrypsin protein but again showed no physiologic effect. In fact, they estimated that the levels of expression that they observed were only about 2% of the level required for protection of the lung in humans, *i.e.*, far below that necessary for a physiologic effect.

The gene for human α1-antitrypsin has been introduced into the liver of normal rats by intraportal injection, where it was expressed and resulted in the secretion of the introduced human protein into the plasma of these rats (Jaffe *et al.*, 1992). However, and disappointingly, the levels that were obtained were not high enough to be of therapeutic value.

These type of results do not demonstrate that adenovirus is able to direct the expression of sufficient protein in recombinant cells to achieve a physiologically relevant effect, and they do not, therefore, suggest a usefulness of the adenovirus system for use in connection with gene therapy.

7. Other Viral Vectors As Expression Constructs

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpes viruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *in vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.* recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was co-transfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

Multigene Constructs and IRES. In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic,

messages. IRES elements are able to bypass the ribosome scanning model of 5′ methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988; Jang *et al.*, 1988). IRES elements from two members of the picornovirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

8. Methods For Gene Delivery

In order to effect expression of intrakine constructs, the expression vector must be delivered into a cell. As described above, the preferred mechanism for delivery is via viral infection where the expression vector is encapsidated in an infectious adenovirus particle.

Several non-viral methods for the transfer of expression vectors into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), polycations (Boussif *et al.*, 1995) and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

In one embodiment of the invention, the expression vector may simply consist of naked recombinant vector. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. For example, Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$

precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding an Intrakine construct may also be transferred in a similar manner *in vivo*.

Another embodiment of the invention for transferring a naked DNA expression vector into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ. DNA encoding a Intrakine construct may be delivered via this method.

In a further embodiment of the invention, the expression vector may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated polynucleotide delivery and expression of foreign DNA *in vitro* has been very successful. Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other

embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression vectors have been successfully employed in transfer and expression of a polynucleotide *in vitro* and *in vivo*, then they are applicable for the present invention. Where a bacteriophage promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacteriophage polymerase.

Another mechanism for transferring expression vectors into cells is receptor-mediated delivery. This approach takes advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993). Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1993). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that an expression vector also may be specifically delivered into a cell type such as lung, epithelial or tumor cells, by any number of receptor-ligand systems, with or without liposomes. For example, epidermal growth factor (EGF) may be used as the receptor for mediated delivery of Intrakine construct in many tumor cells that exhibit upregulation of EGF receptor. Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties.

In certain embodiments, gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene therapy refers to the isolation of cells from an animal, the delivery of a polynucleotide into the cells, *in vitro*, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary

culture of cells and tissues. Anderson *et al.*, U.S. Patent 5,399,346, and incorporated herein in its entirety, disclose *ex vivo* therapeutic methods. During *ex vivo* culture, the expression vector can express the Intrakine construct. Finally, the cells may be reintroduced into the original animal, or administered into a distinct animal, in a pharmaceutically acceptable form by any of the means described below.

For therapy of a human HIV or AIDS patient, the practice of the present invention would include obtaining peripheral blood or bone marrow from the patient. Peripheral blood lymphocytes or stem cells would then be isolated and stimulated. The intrakine gene and any further genetic material as discussed herein would then be introduced into the isolated cells by a transfer system as described above. It is contemplated that a retroviral infection system offers certain advantages. The transduced cells are then expanded in cell culture and re-infused into the patient. Treatment frequency as well as number of cells re-infused in each treatment would depend on the white blood cell count of the patient, and would be determined by the practitioner on an individual basis. It is contemplated, however, that treatments would be required at three to six month intervals.

9. Therapeutic composition

Where clinical application of an expression vector according to the present invention is contemplated, it will be necessary to prepare the complex as a pharmaceutical composition appropriate for the intended application. Generally this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate salts and buffers to render the complex stable and allow for complex uptake by target cells.

Aqueous compositions of the present invention comprise an effective amount of the expression vector, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with

the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The expression vectors and delivery vehicles of the present invention may include classic pharmaceutical preparations. Administration of therapeutic compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration will be by orthotopic, intradermal, intraocular, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients.

The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc.* Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components of the pharmaceutical composition are adjusted according to well known parameters.

The immunopathologic effects of HIV infection are directly related to the interaction of the virus with cells that carry the high affinity receptors for the virus. The immunological abnormalities in patients with AIDS include selective T cell deficiency, decreased in vitro

lynphocyte proliferative responses and the presence of antilymphocyte antibodies. Thus HIV infection results in a depleted lymphocyte concentration. Lymphocyte measurement of patients manifesting AIDS are well known in the art, it is contemplate that the compositions of the present invention could be used to boost the active lymphocyte content of patients that are HIV positive and patients that manifest AIDS. In this respect the therapeutic compositions of the present invention may be employed in the alleviation of the symptoms of AIDS and also in conferring resistance to HIV infection.

As discussed earlier there are several ways in which HIV infection may directly result in the destruction of T4 cell function including infection and destruction of progenitor cells that are responsible for the propagation of the lymphoid cell pool. The present invention thus contemplates methods of treating HIV infection by providing autologous and heterologous bone marrow transplants comprising cells containing the genetic constructs of the present invention.

Adoptive immunotherapy is a therapeutic regimen involving the isolation and *in vitro* cloning and expansion of immunologically active cells from a donor. The expanded, therapeutically active cells are provided to a patient to obtain a therapeutic effect. If the donor is the patient, the transfer is "autologous." If the donor is distinct from the patient, the transfer is "heterologous."

In autologous bone marrow transplantation using the present invention contemplates the use of lymphocytes from patients that are seropositive but have not yet developed the characteristics of HIV infection and that do not have CCR expression at the cell surface and manipulates these cells to express intrakines. These cells are then transplanted into the patient thereby conferring resistance to HIV infection in that patient.

Heterologous bone marrow transplant using the present invention is also contemplated. In such cases lymphocytes from an HLA matched donor are manipulated to express intrakines. Such a bone marrow transplant will be useful in patients with HIV infection whose immune system has been compromised.

Bone marrow may be obtained from normal volunteer donors, normal donors for bone marrow transplantation, or from ribs excised at the time of cardiothoracic surgery of patients with no evidence of hematological disease. In preparing human mononuclear cells, an aliquot of marrow is layered into a receptacle such as a centrifuge tube. Initially, mononuclear cells may be obtained from a source of bone marrow, *e.g.*, tibiae, femora, spine, ribs, hips, sternum, as well as the humeri, radi, ulna, tibiae, and fibulae. Additionally, these cells also can be obtained from

cord blood, peripheral blood, or cytokine-mobilized peripheral blood. Other sources of human hematopoietic stem cells include embryonic yolk sac, fetal liver, fetal and adult spleen, and blood.

The marrow layer is centrifuged to produce a pellet of red cells at the bottom of the tube, a clear layer of media, an interface layer which contains the mononuclear cells and a plasma medium layer on top. The interface layer may then be using for example suction. Centrifugation of this layer at 1000g ultimately yields a mononuclear cells pellet. This pellet may then be resuspended in a suitable buffer for cell sorting by FACS.

To transduce autologous T lymphocytes so that they produce the bicistronic chemokine constructs of the present invention, a blood sample of approximately 200 cc/sample, is isolated from the subject. Lymphocytes are isolated from the blood sample and reared under appropriate conditions following standard protocols as exemplified by Janda et al., Manual of Clinical Microbiology, 5th Edition, American Society for Microbiology, Washington, DC, Chapter 19, p 137; (incorporated herein by reference). In this manner approximately 10^{11} lymphocytes may be isolated from culture after approximately two weeks.

Isolated cultured lymphocytes are transduced with a retroviral or other vector as described herein above such that they will produce the intrakine and secreted forms of the desired chemokyne. These lymphocytes may then be reinfused, or injected, back into the host subject in a pharmaceutically acceptable carrier such that a dose of about 10^9 lymphocytes is delivered. The dosage may be readministered at intervals ranging from 2 weeks to 6 months or one year as desired depending on the state of the subject's immune system. For example, as a subjects WBC is reduced due to HIV infection, the transduced lymphocytes or stem cells may be infused to maintain acceptable levels of HIV immune lymphocytes and to increase circulating chemokynes to inhibit further infection and to reduce the danger of secondary infections in conjunction with other means of HIV therapy.

Of course it is understood that the compositions of the present invention may also be used in combination with traditional therapies for example, those therapies involving zovidovudine (AZT). This is one of a class of nucleoside analogues known as dideoxynucleosides which block HIV replication by inhibiting HIV reverse transcriptase.

11. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Methods

Construction of Expression Vectors. The human MIP-1α and RANTES genes were PCR™-amplified from the cDNA of peripheral blood mononuclear cells (PBMCs). The MIP-1α and RANTES genes were then linked with a KDEL (SEQ ID NO:7) sequence, SEKDEL, ~~SEQ ID NO:7~~ (SEQ ID NO: 6), by PCR™ reactions (Marasco *et al.*, 1993). The native MIP-1α and RANTES genes, and their mutants were cloned into expression vectors, respectively (FIG. 2). All of the constructs were confirmed by DNA sequencing (DNA sequencing core facility of Wake Forest University).

Detection of Protein Expression and Immunofluorescent Staining. To label and precipitate recombinant proteins, cells were radiolabeled with ^{35}S-cysteine and precipitated with antibodies (Chen *et al.*, *Proc. Natl. Acad. Sci. USA* , 91:2932-5936, 1994). After heat denaturation the protein samples were analyzed by electrophoresis on SDS-polyacrylamide gels and visualized by a phosphorimager. For flow cytometric assay, 6×10^5 cells were incubated for 1 hour with a primary antibody, followed by incubation with a fluorescein-conjugate. The cells were analyzed on a Becton Dickinson FACScan. Indirect immunofluorescent staining was performed as described (Chen *et al.*, 1994).

Syncytium Formation Assay. HeLa-T4$^+$ cells grown on dishes (about 50% confluence) were co-transfected pCMV-CCR5 with different amounts of chemokine expression plasmid DNA using a Calcium Phosphate System (Promega). At the same time, HeLa cells were transfected with T- or Mϕ-tropic HIV-1 envelope protein expression vector. 48 hr later, the transfected HeLa cells expressing the envelope proteins (2×10^5) were co-cultured with co-transfected HeLa-T4$^+$ cells (1×10^5). After 12 to 24 hr co-cultivation, syncytia in each well were counted after violet crystal staining.

Generation of Retroviral Packaging Cell Lines and Gene Transfer. The retroviral pLNCX vectors containing the MIP/K or RANTES/K genes (FIG. 2) were transfected into the amphotropic packaging cells (PA317), and the culture medium of the transfected cells was then used to infect the PA317 cells. 48hr later, the infected packaging cells were selected with a G418 (800 μg/ml) containing medium for two to three weeks. The G418-resistant colonies were subcloned and characterized by genetic analysis to ensure the entire incorporation of the genes into the chromosome of the packaging cells. To generate human PBLs, PBMC from healthy individuals were separated on a Ficoll-Hypaque density gradient, and nonadherent PBLs were stimulated in a culture medium (RPMI-1640/20% FCS supplemented with rIL-2 (1,000 IU/ml) (Chiron, CA), anti-CD3 (5 ng/ml) (PharMingen, CA) and PHA (5 μg/ml)) for 48 hr. The stimulated PBL cells were then transduced by co-culture with the recombinant packaging cells in the culture medium containing protamine sulfate (5 μg/ml) for 48 hr. The transduced PBLs were harvested and cultured for one day, followed by transduction in the supernatant of the recombinant packaging cells for additional two days. After final transduction, the PBLs were expanded in the culture medium for several days.

HIV-1 Infection and RT Assay. PBLs in the RPMI medium supplemented with 10% FCS and rIL-2 (1,000 IU/ml) and anti-CD3 (5 ng/ml) were infected with 600 half-maximal tissue-culture infectious dose units ($TCID_{50}$) or 20,000 cpm RT activities of Mϕ- or T-tropic HIV-1 viruses, respectively. After 4-hr incubation at 37°C, the infected PBLs were then washed once and resuspended in the culture medium, and the RT activities in these cultures were then determined as described (Chen *et al.*, 1996).

EXAMPLE 2

Chemokine Targeting to The Endoplasmic Reticulum Lumen

A mutated chemokine was targeted to the lumen of the ER of lymphocytes to intracellularly block the transport and surface expression of newly synthesized CCR5, which resulted in the phenotypic CCR5 knock-out (FIG. 1).

MIP-1α and RANTES, and their mutated genes (MIP1-K and RANTES-K) linked with a retention signal sequence for residential soluble ER proteins (KDEL) (SEQ ID NO: 7) (Munro and Pelham, 1987) were cloned into an expression vector (pRc/CMV) with a Neo marker, (FIG. 2). A bi-cistronic vector for co-expression of both the native and mutated chemokines was also constructed to inhibit HIV infection both intracellularly and extracellularly. Expression of these two forms of

chemokines inhibits viral infection to susceptible cells by secreting chemokines as well as to phenotypically knock-out CCR5 by intracellular binding (FIG. 2).

To determine the expression and localization of the native and mutated chemokines, radiolabeling and immunoprecipitation studies were performed. COS cells were transfected with plasmid DNA, and 48 hr later radiolabeled with ^{35}S-cysteine. The cell lysate and culture medium were then immunoprecipitated with an anti-human MIP-1α. An 8 Kd protein band corresponding to MIP-1α was found in both the culture medium and cell lysates of the pCMV-MIP1-transfected cells, but not in the control cells.

In pCMV-MIP1-K-transfected cells, the MIP-1α proteins were predominately found in the cell lysate, not in the culture medium. To further determine the cellular retention of MIP1-K, the transfected cells were pulse-radiolabeled for 30 min., chased for various times, and then immunoprecipitated. The MIP1-K proteins were found to be stably retained intracellularly with a half-life of over 4 hr. In the bi-cistronic expression vector-transfected cells, both the MIP1 and MIP1-K proteins were co-expressed.

The localization of the native and mutated chemokines was further examined by immunofluorescent staining. An ER staining pattern was observed throughout the cytoplasm in the cells transfected with pCMV-MIP1-K, while a perinuclear Golgi staining pattern was seen in the cells transfected with pCMV-MIP1. The native and mutated RANTES proteins were also expressed in the transfected cells in a similar manner with the native and mutated MIP1 proteins. Thus, these results indicate that the mutated chemokines were effectively expressed, and stably retained in the ER, while native chemokines were secreted out of the cells.

EXAMPLE 3

Effects of Intrakines on CCR5 surface Expression

To examine the effects of intrakines on CCR5 surface expression, a vector for expression of CCR5 tagged with an HA epitope (Liu *et al.*, 1996; Field *et al.*, 1988) was constructed (FIG. 2). A flow cytometric assay was used to determine the surface expression of HA-CCR5 on the cells co-transfected with the intrakine expressing vectors.

As shown in FIG. 3B, when transfected with pCMV-HA-CCR5 alone, the cell surface expression of HA-CCR5 was detected in 64.7% cells. However, when co-transfected with increasing amounts of pCMV-MIP1-K, the cell numbers with positive surface staining for HA-CCR5 were dramatically decreased (FIG. 3C and FIG. 3D). Co-transfection with

pCMV-MIP1-K did not interfere with the cell surface expression of an unrelated murine leukemia virus envelope protein (Matsuoka *et al.*, *J. Biol. Chem.*, 269:22565-22573, 1994). This result demonstrates that the CCR5 surface expression is blocked by intrakines.

To determine whether the blockade of the HA-CCR5 surface expression is a result of the intracellular binding of MIP1-K to newly synthesized HA-CCR5, a co-immunoprecipitation assay was performed. In the cells co-transfected with pCMV-MIP1-K and pCMV-HA-CCR5, the MIP1-K proteins were co-precipitated by the anti-HA antibody. The specificity of the co-immunoprecipitation was further confirmed based on the observation that the anti-HA antibody did not precipitate the MIP1-K in the cells transfected with pCMV-MIP1-K alone, or co-transfected with a vector over-expressing irrelevant proteins such as viral PTV G proteins (Matsuoka *et al.*, 1994; Chen *et al.*, 1994).

It was noted that no distinguishable band of the CCR5 proteins appeared on SDS-PAGE, although the specific co-immunoprecipitation and flow cytometric data indicated the HA-CCR5 expression in the cells. Heterogeneous patterns of glycosylation or other undefined properties may contribute to the anomalous migration of these receptors on polyacrylamide gels, as observed in previous studies (Liu *et al.*, 1996; Strader *et al.*, 1994). Taken together, these results indicate that the ER-retained intrakines bind newly-synthesized CCR5 molecules and prevent their transport to the cell surface.

EXAMPLE 4

Effects of Intrakines on CCR5

To further examine the effects of intrakines on CCR5, a sensitive CCR5/CD4-mediated syncytium formation assay was performed (Deng *et al.*, 1996). The transformed cells expressing CD4 (HeLa-T4$^+$)(Marasco *et al.*, 1993) were co-transfected pCMV-CCR5 with different amounts of pCMV-MIP1-K, and 48 hr later, co-cultured with the HeLa cells expressing Mϕ (ADA and YU2) or T (IIIB)-tropic HIV-1 envelope proteins (Choe *et al.*, 1996) for 12 to 24 hr.

As shown in FIG. 3E, in the co-cultures with the cells expressing pCMV-MIP1-K, significant inhibition of Mϕ-tropic envelope-mediated syncytium formation was observed. When co-transfected with increasing amounts of pCMV-MIP1-K, Mϕ-tropic envelope-mediated syncytium formation was almost completely inhibited. However, the T-tropic envelope-mediated syncytium formation was not inhibited by transfection with pCMV-MIP1-K. Inhibitory effects on the syncytium formation of Mϕ-tropic envelope proteins at various degrees

were also observed in the cells co-transfected with pCMV-MIP1, pCMV-MIP1/K, pCMV-RANTES, pCMV-RANTES-K, or pCMV-RANTES/K (FIG. 3E). The inhibition of syncytium formation by expression of native (secreted) MIP-1 or RANTES may be due to the partial saturation of the binding site on CCR5.

To evaluate potential therapeutic applications, the bi-cistronic expression cassettes (MIP1/K and RANTES/K) were cloned into a murine retroviral shuttle vector, pLNCX (Miller, 1992) (FIG. 2). Transformed packaging cell lines (PA317) (Miller, 1992) producing recombinant retroviruses containing the chemokine genes were generated, and characterized. Fresh human PBLs were then transduced with the MIP/K gene by co-culture with the transformed packaging cells. After transduction, the specific DNA fragments corresponding to the chemokine genes and IRES sequence were amplified from the genomic DNA of transduced PBLs by PCR™. Expression of the MIP-1 and MIP1-K proteins was detected in the transduced PBLs, but not in untransduced PBLs by radiolabeling and immunoprecipitation assays.

The transduced or mock-transduced PBLs were then infected with Mϕ- or T-tropic HIV-1 isolates, respectively, and the viral production in the cell cultures was examined by a reverse transcriptase (RT) assay (Chen *et al.*, 1994). The transduced or untransduced PBLs (2×10^5) were equally infected with 600 TCID50 or 20,000 cpm RT of several Mϕ- or T-tropic HIV1 viruses for 4 hr, and then replaced with the fresh culture medium containing 1,000 IU/ml of rIL-2 and anti-CD3 (5 μg/ml). Every three to four days, cell numbers in each well were counted, and the culture medium was subjected to RT assays. RT activities (10^5 cells/ml) in duplicate wells after subtracting the background were calculated. Only low levels of RT activities were detected in the cultures of transduced PBLs infected with Mϕ-tropic viruses, but high levels of RT activities detected in the control PBL culture. However, T-tropic HIV-1 viruses were able to infect both the transduced and mock transduced PBLs. These results indicate that human PBLs transduced with intrakine genes are resistant to Mϕ-tropic HIV-1 infection.

EXAMPLE 5

Genetic Modification of Lymphocytes Expressing Intracellular CXC-Chemokines to Inactivate CXR4 Receptor for HIV-1 Gene Therapy

Fusin/CXC-chemokine receptor (CXR)-4 with seven-transmembrane segments is a co-receptor of T-tropic human immunodeficiency virus (HIV)-type1 which is required for the fusion and entry to CD4-positive lymphocytes. Because of the critical role of CXR4 for HIV-1

infection, the inventors hypothesized that lymphocytes with phenotypic knock-out of CXR4 would be resistant to HIV-infection. In this study, stromal cell-derived factor-1 (SDF), a biological ligand for CXR4, was genetically modified to target the lumenal endoplasmic reticulum (ER) of lymphocytes (FIG. 4A). As a result, the intracellularly retained SDF bound the newly synthesized CXR4 and prevented its transport to the cell surface.

The lymphocytes with the phenotypic CXR4 knock-out were resistant to T-tropic HIV-1 infection. In addition, co-expression of the secreted and ER-retained chemokines was achieved for additional inhibition of viral infection. In summary, this novel approach uniquely targeting at the CXR4 co-receptor should have a significant application for HIV-1 gene therapy.

Genetic defects in CC-chemokine receptor (CCR)-5, the co-receptor for macrophage-tropic HIV-1, were found to be responsible for natural resistance of some individuals to HIV-1 infection. These data suggest that the knock-out of CXR4 in lymphocytes may have a therapeutic implication. In the inventors' and other studies, blockade of cell surface expression of a membrane protein has been accomplished by intracellular binding of intrabodies or other molecules targeted to the ER (Marasco *et al.*, 1993; Chen *et al.*, 1995a; Chen *et al.*, 1995b; Buonocore and Rose, 1990). In this study, a mutated SDF was generated in order to target the lumen of the ER of lymphocytes for intracellular blockade of the transport and surface expression of newly synthesized CXR4s (FIG. 4A). The genetically modified lymphocytes without the co-receptor CXR4 on the cell surface were found to be resistant to T-tropic HIV-1 infection.

The SDF-1 gene was cloned from the cDNA of mouse spleen, with only one amino acid difference with human SDF-1, and then genetically linked with a retention signal sequence for residential soluble ER proteins (KDEL) (SEQ ID NO:7) (Munro and Pelham, 1987). The SDF gene and its derivative (SDF-K) were then cloned into an expression vector (pRc/CMV) with a Neo selection marker (FIG. 4A). A bi-cistronic vector for co-expression of the native and mutated chemokines was also constructed in order to inhibit viral infection to susceptible cells by extracellular secretion of chemokines, as well as to phenotypically knock-out CXR4s by intracellular binding (FIG. 4A).

Resistance to T-tropic HIV-1 infection of transduced lymphocytes is shown in FIG. 4B. The transduced cells expressing SDF, SDF-K or untransduced Jurkat cells (2×10^5) were equally infected with 600 TCID50 T-tropic HIV-1 virus, for 4 hr, and then placed with the fresh culture

medium. RT activities in duplicate wells after subtracting the background level are presented (FIG. 4B).

EXAMPLE 6

Effect of Intracellular SDF-Intrakine Expression on CXCR4 Surface Expression

The SDF-1α gene and modified gene linked with an ER retention signal sequence (KDEL) (SEQ ID NO: 7) (Marasco *et al.*, 1993) were cloned into expression vectors (FIG. 2). Constructs containing an influenza hemagglutinin (HA) tag (Field *et al.*, 1988) were also generated to facilitate protein detection. Expression of the SDF-1 and SDF-K proteins was determined by radiolabeling and immunoprecipitation analysis. A SDF-1 protein band precipitated by an antibody to the HA tag was found in both culture medium and lysate of HeLa cells transfected with the construct containing the native SDF-1 gene with the HA tag (pCMV-SDF-HA). No corresponding protein band was detected in the cells transfected with a control plasmid. However, the SDF-K proteins were predominately found in the cell lysate, not in the culture medium of the transfected cells, suggesting that the native SDF-1 was secreted out of the cells, but the modified SDF-K (SDF-intrakine) was retained intracellularly. To further demonstrate the intracellular retention of SDF-K, pulse-chase experiments were performed. It was shown that the native SDF-1 was efficiently secreted from the cells, while the SDF-intrakine was stably retained intracellularly with a half-life greater than 4 hr.

Inhibition of CXCR4 surface expression by SDF-intrakine expression was then examined using a sensitive CXCR4/CD4-mediated syncytium formation assay (Bluel *et al.*, 1996; Marasco *et al.*, 1993). The CXCR4 and CD4-positive HeLa-T4 cells seeded on 12-well plates were co-transfected with a IIIB envelope expressor (Marasco *et al.*, 1993) and SDF expression vectors. In the cells transfected with the envelope expressor only or co-transfected with the control vector, extensive syncytium formation was observed. However, in the cells co-transfected with pCMV-SDF-K, no or only few polykaryons were formed. Co-transfection with pCMV-SDF partially inhibited the syncytium formation. Three repeated studies showed consistent inhibitory effects of SDF-intrakine on syncytium formation. A co-immunoprecipitation assay was further performed to determine the possible intracellular binding of the intrakine and CXCR4. The SDF-intrakine was co-precipitated from the CXCR4-positive HeLa cells (Bluel *et al.*, 1996) transfected with pCMV-SDF-K using an anti-CXCR4 antibody, suggesting the association of the SDF-intrakine

and-CXCR4. In addition, the surface expression of CXCR4 on the transduced lymphocytes expressing SDF-K were dramatically decreased as demonstrated by flow cytometric assays. Taken together, the results from these transient assays suggest that SDF-intrakines bind newly synthesized CXCR4 and block its transport to the cell surface.

Construction of expression vectors: The mouse SDF-1α gene with a single amino acid difference with the human gene was PCR-amplified from the cDNA of mouse spleen with the primers

5'-TTAAGCTTCGCGCCATGAACGCCAAGGTC-3' (SEQ ID NO:2) (P 1)

and 5'-TTTGCGGCCGCTTACTTGTTTAAAGCCTTCTCCAGGT-3') (SEQ ID NO:3)(Nagasawa *et al.*, 1994; Shirozu *et al.*, 1995). An HA tag sequence (identified herein as SEQ ID NO:1) was linked to the SDF-lα gene by a PCR reaction with the primer designated as SEQ ID NO:2 and

5'-

TTTTCTAGATTAAGCATAATCTGGAACATCATACGGATACTTGTTTAAAGCCTTCTCC AG-3') (SEQ ID NO:4). The SDF-1α or SDF-lα-HA gene was then linked with an ER retention signal (SEKDEL, SEQ ID NO:6) by a PCR reaction with the primers (P 1 and 5 '-TTTTCTAGATTACAGCTCGTCCTTCTCGCTAGCATAATCTGGAACATCATA-3') (SEQ ID NO:5). These DNA fragments were digested with HindIII/XbaI and cloned into an expression vector (pRc/CMV) Marasco *et al.*, 1993). The SDF-lα-KDEL fragment was further cloned into the retroviral vector pLNCX (Chen *et al.*, 1997), and the resultant construct was designated as LNCX-SDF-K/Neo. A truncated nerve growth factor receptor (ΔNGFR) gene PCR-amplified from the MN vector DNA (Phillips *et al.*, 1996; Rudoll *et al.*, 1996) was cloned into LNCX-SDF-K/Neo by replacing the neomycin selection marker. All of the constructs were identified by restriction enzyme digestion and confirmed by DNA sequencing.

EXAMPLE 7

Generation and Evaluation of Intrakine-Expressing Lymphocyte Lines

To further determine the effects of SDF-intrakine expression, two CD4/CXCR4+-immortal lymphocyte lines, Jurkat and Molt-4 (Marasco et al., 1993; Daniel *et al.*, *J. Virol.* 62,4123-4128, 1988), were transfected with various expression vectors by electroporation, followed by G418 selection (Chen et al, 1994a; Chen *et al.*, *Nature* 385, 78-80, 1997). Incorporation of the SDF

vectors into the transduced lymphocytes was demonstrated by genomic PCR amplification. Transcription of the incorporated SDF1 or SDF-K genes was also detected by reverse transcriptase (RT)-PCR. Moreover, expressed SDF-1 was found to be secreted out of the transduced lymphocytes, while SDF-intrakine was retained inside the lymphocytes. The effects of SDF-intrakine on CXCR4 surface expression of the transduced lymphocytes were examined by flow cytometric assay (Chen *et al.*, 1994a). Surface expression of CXCR4 was detected on the Molt-control, but dramatically decreased on Molt-SDF-K. In contrast, comparable levels of CD4 surface expression were detected in both the parental and transduced Molt. The biological features of the transduced lymphocytes, including cell proliferation and DNA synthesis rates, were found to be similar to the parental lymphocytes.

To determine whether SDF-intrakine expression would lead to the resistance of the transduced lymphocytes to T-tropic HIV-1 entry, an envelope-complementation assay was used. In this assay, HIV-1 envelope glycoproteins expressed *in trans* complement a single round of replication of an envelope-deleted provirus encoding the chloramphenicol acetyltransferase (CAT) gene (Chen et al., 1994b; Choe *et al.*, 1996) .Recombinant viruses that were pseudotyped with the envelope glycoproteins derived from the T-tropic (IIIB) virus were produced, and the efficiency of the recombinant pseudovirus entry was assessed by measuring CAT activity in the cells 60 hr after infection. High levels of CAT activity in the control lymphocytes were detected after infection with the recombinant IIIB virus, but the CAT activity levels in the transduced lymphocytes expressing SDF-intrakine were dramatically decreased. The partial inhibition of virus entry of the transduced lymphocyte expressing the native SDF-1 may be due to the partial saturation of the binding site on CXCR4 during and after SDF-1 secretion.

To further examine the effect of SDF-intrakine, transduced or control Molt lymphocytes were infected with 20,000 cpm RT of T-tropic IIIB viruses. Extensive syncytium formation in the Molt-control was observed six days postinfection, while only a few syncytia were observed in the Molt-SDF-K cell culture. In agreement, high levels of RT activities were detected in the Molt-control culture, but only low levels of RT detected in the Molt-SDF-K culture. Molt-SDF secreting the native SDF-1 were partially resistant to virus infection. To confirm the result, transduced or untransduced Jurkat lymphocytes were also infected with the IIIB virus, and the dramatic anti-HIV effects of SDF-intrakine were also observed in the transduced cells. Thus, these results indicate that the transduced lymphocytes expressing SDF-intrakine are viable and resistant to T-tropic HIV-I infection.

Generation of transformed lymphocyte lines: About $1x10^6$ Molt-4 and Jurkat human immortal lymphocytes were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), and transfected with 10 ug of plasmid DNA by electroporation (Yang *et al.*, *Nature Biotechnology* 15, 46-51, 1997). 48 hr later, the transfected lymphocytes were then selected in the RPMI-1640/10% FCS containing 800 ug/ml of G418 (Gibco-BRL) on 24-well plates for two to three weeks. The G418-resistant cells were picked and subcloned by limited dilution as described elsewhere (Chen *et al.*, 1994a) (incorporated herein by reference).

Envelope-complementation assay. 293 cells grown on 10-cm dishes were co-transfected with pHXBΔenvCAT (20 ug) and an envelope protein expressor (5 ug) Chen *et al.*, 1994b; Choe *et al.*, 1996). 48 hr later, the culture medium was harvested, and RT activities in the supernatant were measured (Yang *et al.*, 1997). The recombinant viruses (20,000 cpm RT activities) were used to infect target cells by overnight incubation, and 60 hr later, the target cells were then lysed and used for determination of CAT activity using a kit (Promega).

Detection of protein expression and flow cytometric assay. To label and immunoprecipitate recombinant proteins, cells were radiolabeled with ^{35}S-cysteine or -Trans for various times, and the cell lysates and culture media were then precipitated with antibodies as described elsewhere (Chen *et al.*, 1996, encorporated herein by reference). After heat denaturation, the protein samples were analyzed by electrophoresis on SDS-polyacrylamide gels and visualized by a Phosphorimager. For flow cytometric assay, 1 x 10^6 lymphocytes were incubated with a primary antibody for 1 hour, followed by incubation with a fluorescein-conjugate. The cells were then analyzed on a Becton Dickinson FACScan.

EXAMPLE 8
Resistance to HIV-1 Infection of Transduced PBLs

The following example demonstrates the ability of SDF-intrakine to confer resistance to HIV-1 infection on human peripheral blood lymphocytes (PBLs). Initially, PBLs were transduced with the recombinant retrovirus containing the SDF-K and Neo selection marker (FIG. 2), and viral infection in the transduced PBLs after Neo selection was dramatically inhibited. However, due to the nonspecific toxicity to primary PBLs, difficulty in reliably assessing the transduction efficiency, and a prolonged process of the Neo selection, a truncated human nerve growth factor receptor (ΔNGFR) which expresses on transduced cells was used as a marker for quantifying the

gene transfer efficiency and isolating transduced PBLs (Phillips *et al.*, *Nature Medicine* 2, 1154-1156, 1996; Rudoll *et al. Gene Therapy* 3, 695-705, 1996). About 7 to 10 percent of the stimulated PBLs were transduced by the recombinant retroviral vector (LNCX-SDF-K/ΔNGFR) or control retroviral vector (MN) that expresses the ΔNGFR marker only, produced from transiently transfected packaging cells (Bing) (Pear *et al.*, *Pro. Nat. Acad. Sci. USA*. 90, 8392-6, 1993: Pear *et al.*, Methods in Molecular Biology: Methods in Gene Therapy (P. Robbins, ed.), (Humana Press, Totowa, NJ), 41-57, 1997). The transduced PBLs were then isolated with an anti-NGFR/anti-IgG-magnetic bead kit (ImmunoTech Inc., Westbreak, ME). After isolation, over 93 percent of the PBL population were positive for the NGFR marker.

To evaluate the anti-HIV-1 effect of SDF-intrakine, the isolated PBLs transduced with either LNCX-SDF-K/ΔNGFR or MN control were infected with an identical IIIB virus inoculum, and the viral production in the cultures was examined by the RT assay. Only low levels of RT activity were detected in the cultures of transduced PBLs, but high levels of RT were detected in the MN (control)-transduced PBL culture. Thus, the SDF-intrakine gene was efficiently transduced into primary human PBLs, and the transduced PBLs were resistant to T-tropic HIV-I infection.

Biological Evaluation of Transduced PBLs

Although several lymphocyte lines expressing SDF-intrakine were shown to have normal biological features, the effect of expression of SDF-intrakine on primary human lymphocytes was examined. Accordingly, fresh human PBLs were transduced with LNCX-SDF-K/ΔNGFR or MN vectors, and the transduced PBLs were isolated. Several biological analyses were carried out. First, the PBLs transduced with either LNCX-SDF-K/ΔNGFR or MN were subjected to flow cytometric analysis. There were comparable levels of surface expression of CD3 and CD4 molecules on the PBLs transduced with LNCX-SDF-K/ΔNGFR or MN.

In contrast, CXCR4 surface expression on the PBLs transduced with LNCX-SDF-K/ΔNGFR was dramatically decreased, when compared to the CXCR4 expression on the PBLs transduced with MN. These results indicate that SDF-intrakine selectively blocks CXCR4 surface expression. Chemotaxis assays were also performed to determine the responsiveness of the transduced PBLs to chemokine stimulation. The responsiveness of PBLs transduced with LNCX-SDF-K to the stimulation of recombinant SDF-1 was significantly decreased, when compared to that of PBLs

transduced with the control MN. However, the PBLs transduced with LNCX-SDF-K or MN were equally sensitive to the stimulation of MIP-1α which binds to CC-chemokine receptors (Baggliolini *et al.*, 1994). These results further demonstrate the selective inactivation of CXCR4 by SDF-intrakine. It was noted that a portion of PBLs transduced with LNCX-SDF-K responded to SDF-1 at a high concentration, probably due to residual CXCR4 on a portion of the PBLs, or an unidentified interaction of SDF-1 with chemokine receptors, other than CXCR4. Third, PBLs transduced with LNCX-SDF-K/ΔNGFR were also shown to normally respond to anti-CD3/CD28 or PHA stimulation in IL-2 production, cell proliferation, and DNA synthesis. Thus, these results suggest that human PBLs expressing SDF-intrakine maintain normal biological activities.

Retroviral vector production and gene transduction. PBMC from healthy individuals were separated on a Ficoll-Hypaque density gradient, and nonadherent PBLs were stimulated in a culture medium (RPMI-1640/20% FCS supplemented with rIL-2 (500 IU/ml) (Chiron, CA) and anti-CD3 (5 ng/ml) (PharMingen, CA) for 48 hr. An amphotropic retroviral packaging cell line, Bing, was used to transiently produce recombinant retroviral vectors. Bing cells were plated on 100 mm dishes at 80% confluence and transfected with 15 ug of LNCX-SDF-K/ΔNGFR or MN plasmid DNA by using a calcium phosphate transfection kit (Promega). 48 hr later, the stimulated PBLs were cultured in the supernatant of the transfected Bing cells containing rIL-2, anti-CD3, and protamine sulfate (5 ug/ml) for 24 to 72 hr at 37°C. The transduced PBLs were harvested and subjected to flow cytometric analysis to detect the NGFR marker on PBLs.

Isolation of transduced PBLs: Anti-mouse IgG magnetic immunobeads (ImmunoTech Inc.), 1 micron spherical particles directly coated with affinity-purified sheep polyclonal antibody directed to the Fc fragment of mouse IgG, were diluted to 1:20 in PBS/0.2% BSA. Separation of the beads from the buffer solution was achieved by placing the tube in a Cobalt-Samarium magnet support (ImmunoTech Inc.) for 5 min. 5 to 20 ug of the anti-human NGFR antibody (Boehringer Mannheim, Indianapolis, IN) were added into 1 to 4 mg of beads in PBS/0.2% BSA and incubated at room temperature for 15 min. After washing with PBS/1.2% BSA, 0.5 mg (50 µl) of the beads were added into 1 ml of 10^7 PBLs in PBS/30% FCS, and incubated 10 min at room temperature. The beads/PBLs solution was then placed in the Cobalt-Samarium magnet support for 10 min, and the supernatants containing the unbound cells were discarded. The beads/PBLs rosettes were washed twice with PBS/0.2% BSA, and resuspended in the culture medium for 24 hr at 37° C.

Chemotaxis assay: $5x10^5$ isolated PBLs transduced with LNCX-SDF-K/ΔNGFR or MN control vector were suspended in 100 ul of the RPMI-1640 medium containing 0.25% human serum albumin, and added to the top chamber of a 5-μm pore polycarbonate Transwell culture insert (Costar, Cambridge, MA). 500 ul of the RPMI-1640/0.25% albumin containing various concentrations of the recombinant human MIP-1α or SDF-1 (R & D System Inc., Minneapolis, MN) were added to the bottom chamber of the Transwell. After 3 hr incubation at 37°C, the cell numbers in the bottom chamber were counted, and percentages of the transmigration are presented after substracting the background (absence of chemokines) transmigration Bleul *et al.*, 1996).

* * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.